

# EXHIBIT F

Exhibit F

## Changing Pattern of Expression of the Epidermal Growth Factor Receptor and Transforming Growth Factor $\alpha$ in the Progression of Prostatic Neoplasms<sup>1</sup>

Howard I. Scher,<sup>2</sup> Alvaro Sarkis, Victor Reuter, David Cohen, George Netto, Daniel Petrylak,<sup>3</sup> Pilar Llanes, Zvi Fuks, John Mendelsohn, and Carlos Cordon-Cardo

Genitourinary Oncology Service, Division of Solid Tumor Oncology [H. I. S., D. P.], Departments of Medicine [H. I. S., D. P., J. M.] and Pathology [A. S., V. R., G. N., P. L., C. C-C.], Urology Service, Department of Surgery [D. C.], Department of Radiation Oncology [Z. F.], Memorial Sloan-Kettering Cancer Center, and Department of Medicine, Cornell University Medical College [H. I. S., D. P., J. M.], New York, New York 10021

### ABSTRACT

The autocrine/paracrine interaction of the epidermal growth factor receptor (EGFr) and transforming growth factor  $\alpha$  (TGF- $\alpha$ ) has been implicated in prostate cancer cell growth and proliferation. To evaluate the role of EGFr and TGF- $\alpha$  in prostate cancer progression, we studied the immunohistochemical staining pattern of EGFr and TGF- $\alpha$  in malignant primary and hormone-independent metastatic prostate lesions. The specimens evaluated included 37 primary carcinomas (34 hormone-naïve and 3 hormone-refractory tumors) and 22 metastases. For each specimen, the pattern of expression was evaluated and staining reactivities graded from 0-3, with 0 representing no staining and 3 representing homogeneous and intense staining. Primary malignant prostate epithelial cells in areas with discrete gland formation showed strong EGFr immunostaining, while stromal cells were generally nonreactive. In untreated primary tumors, TGF- $\alpha$  expression was primarily in the stroma, while epithelial cells were weakly positive in several cases. Malignant epithelial cells adjacent to neural elements that stained positive for TGF- $\alpha$  was frequently observed. A homogeneous staining pattern for EGFr was noted in 17 (89%) of 19 evaluable androgen-independent-refractory metastases, while TGF- $\alpha$  expression was found in 14 (78%) of 18 evaluable cases. Overall, 14 of 18 androgen-independent metastases coexpressed the receptor and the ligand. These results suggest that, unlike primary prostate tumors where a paracrine relationship between EGFr and TGF- $\alpha$  appears to predominate, the potential for autocrine stimulation may exist in the majority of metastatic androgen-independent

tumors. Furthermore, the changing pattern of expression as the disease evolves from the localized hormone-naïve to metastatic androgen-independent condition suggests that strategies aimed at blocking this growth factor pathway may be of therapeutic importance for androgen-independent disease.

### INTRODUCTION

Growth factors that act via transmembrane tyrosine kinases are believed to contribute to prostate cancer cell growth and proliferation. These include TGF- $\alpha$ ,<sup>4</sup> EGF, insulin-like growth factor(s), and transforming growth factor  $\beta$  (1-3). The evidence that the EGFr pathway is important in prostate cell growth includes the observation that: (a) human prostate tumor-derived cell lines synthesize TGF- $\alpha$  in culture (4-7), (b) prostatic tumor cells express the EGFr *in vitro* and *in vivo* (8, 9), and (c) TGF- $\alpha$  or EGF added to cultured cells are mitogenic (4, 5, 10).

EGFr have been studied in human prostate cancers by biochemical analysis of tissue homogenates (11-13), immunohistochemistry (14-16), and *in situ* hybridization (17-19). Most studies have focused on untreated primary and in some cases, regional lymph node metastases from patients who have not been exposed to hormonal therapy. Few data are available on the expression in metastatic lesions from patients with hormone-independent tumors. In a previous study dealing with primary tumors, we reported EGFr expression in the majority of tumor cells with no correlation between expression and grade (20). In most cases, TGF- $\alpha$  expression was present in the stroma; however, coexpression of EGFr and TGF- $\alpha$  was noted in a small subset of lesions (20). A similar staining pattern for TGF- $\alpha$  was observed by Harper *et al.* (21). The present study extends our previous observations to include recurrent primary and metastatic tumors from patients with androgen-independent disease.

### MATERIALS AND METHODS

**Patient Population.** Prostatic cancers from 59 patients were evaluated. These individuals were categorized on the basis of disease extent using the TNM system (22) and hormonal status. The latter included: (a) naïve (no prior hormonal therapy) and (b) androgen-independent (progressive disease despite castrate levels of serum testosterone).

**Tissues.** The 59 malignant prostatic tumors included 34 primary hormone-naïve and 25 androgen-independent (3 primary and 22 metastases) lesions. Untreated primary material

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<sup>2</sup>To whom requests for reprints should be addressed, at Memorial Sloan-Kettering Cancer Center, 1275 York Avenue, New York, NY 10021.

<sup>3</sup>Present address: Division of Medical Oncology, Columbia Presbyterian Medical Center, New York, NY.

<sup>4</sup>The abbreviations used are: TGF- $\alpha$ , transforming growth factor  $\alpha$ ; EGF, epidermal growth factor; EGFr, EGF receptor; DAB, diaminobenzidine.

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Fig. 1 Expression of EGFr in hormone-naïve primary prostate cancer. EGFr staining was predominantly observed in areas of glandular differentiation.

was obtained from patients undergoing radical prostatectomy for clinically localized disease. Patients with androgen-independent disease had demonstrated progression of disease despite castrate levels of testosterone. They had progressed on either a gonadotropin-releasing hormone analogue or orchiectomy plus an antiandrogen. Those who were not surgically castrated were continued on medical therapies to maintain castrate levels of testosterone. In these cases, tissues were obtained by transrectal biopsy in patients with locally progressive disease, or from metastatic lesions by computerized tomographic guided biopsy. The metastatic sites included lymph nodes in 15 cases, hepatic lesions in 5 cases, 1 adrenal metastasis, and 1 bone biopsy. Informed consent was obtained for biopsies of hormone-independent lesions under a protocol approved by the Institutional Review Board.

Fresh tissues were embedded in OCT compound in cryomolds (Miles Laboratories Inc., Naperville, IL), immersed in isopentane precooled in liquid nitrogen, and stored at  $-70^{\circ}\text{C}$  until needed. Tissues were first evaluated for routine histology and consecutive sections were used for immunohistochemical analysis.

**Reagents.** mAb 528 detecting the EGFr was supplied by Dr. John Mendelsohn. It was provided as a purified reagent and used at approximately  $20\text{ }\mu\text{g/ml}$  in PBS containing 2% BSA (Hybritech Inc., San Diego, CA). This antibody binds the EGF receptor with a  $K_d$  of 2 Nm, comparable to the natural ligand, can precipitate the EGFr, competitively blocks the binding of  $^{125}\text{I}$ -labeled EGF to the receptor, and is internalized by A431 cells (23, 24). It can inhibit EGF/TGF- $\alpha$ -induced activation of receptor tyrosine kinase and inhibit EGF-induced proliferation *in vitro* and *in vivo* (25). Antibody 4.4, an IgG<sub>1</sub> monoclonal to TGF- $\alpha$ , was obtained from Oncogene Science, Inc. (Manhasset, New York, NY). Purified mouse mAb IgG<sub>1</sub> class directed against the cell surface antigen anthranilate synthase of *Escherichia coli* (bcr-25; Oncogene Science, Inc.) was used as a negative control. All antibodies were used at the same working dilutions ( $20\text{ }\mu\text{g/ml}$ ) in PBS containing 2% BSA.

Purified mAbs against cytokeratins and other intermediate filaments (e.g., vimentin) were used at the same concentration

( $20\text{ }\mu\text{g/ml}$ ; Signet Laboratories Inc., Dedham, MA) as positive controls. The secondary antibodies were biotinylated horse anti-mouse IgG (heavy + light chains specific) affinity purified antibodies (1:100 dilution in PBS) and the tertiary reagents were avidin-biotin-peroxidase complexes (1:100 dilution in PBS; Vector Laboratories Inc., Burlingame, CA). DAB was used as chromogen (5 mg DAB tetrahydrochloride in 100 ml PBS with  $100\text{ }\mu\text{l}$  0.3% hydrogen peroxide).

**Immunohistochemical Technique.** Frozen tissue sections were used for the present study. EGFr and TGF- $\alpha$  expression was assessed using a standard avidin-biotin-immunoperoxidase technique as described previously (26). First, hematoxylin and eosin stains of each block were performed to confirm the histology. Once confirmed, cryostat-cut tissue sections ( $4\text{-}\mu\text{m}$  thick) were cut and stored at  $-70^{\circ}\text{C}$  until needed. Slides were cleaned in 95% alcohol and subbed in poly-L-lysine to ensure that the tissue sections adhered. Sections were air dried at room temperature for 1 h, fixed in cold acetone ( $4^{\circ}\text{C}$ ) for 10 min, and washed in phosphate buffer sodium chloride solution (0.85%).

The primary antibody was incubated for 1 h after the appropriate dilution having been previously established. Sections were washed with PBS and incubated for 45 min with secondary horse anti-mouse biotinylated antibodies, previously titrated for optimal dilutions. This was followed by the avidin-biotin-peroxidase (Vector Laboratories, Inc.) complexes (1:100 in PBS) and DAB solution as chromogen (see above). The DAB solution was filtered and incubated with the tissue sections for 6–12 min. After treatment, the sections were washed with distilled  $\text{H}_2\text{O}$ , counterstained with hematoxylin, and mounted with permount. Vigorous washes with PBS were carried out between steps.

**Controls.** Frozen normal tissues expressing the appropriate antigen(s), e.g., skin, were used for titration of the reagents as well as positive controls. Negative controls included substitution of the primary antibody by a similar antibody of the same species and class (bcr-25, see above).

**Scoring.** All samples were scored for positivity by independent reviewers (V. E. R. and G. N.) who were blinded to the clinical status. They were graded for immunoreactivity by estimating the percentage of positive tumor cells as follows: minimal/no immunoreactivity (0–1+) = 0–5% of the epithelial tumor cells positive; heterogeneous (2+) = 5–70% of the epithelial tumor cells positive; and homogeneous (3+) = >70% of tumor cells positive. The pattern of staining, including the cell type stained (epithelial *versus* stromal), was also assessed.

## RESULTS

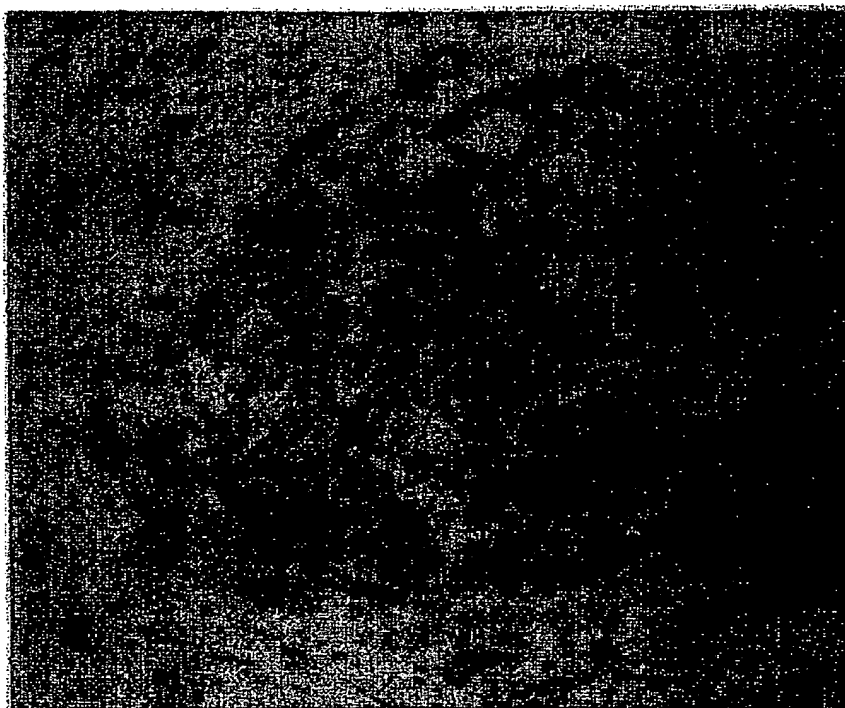
Expression of EGFr using mAb 528 and TGF- $\alpha$  using mAb 4.4 on frozen histological sections was reproducible. Different patterns of expression were observed on tissues from primary hormone-naïve and hormone-refractory metastatic sites.

**Hormone-naïve Primary Tumors.** Immunohistochemical staining of malignant epithelial cells for EGFr was observed on 31 (91%) of the 34 untreated primary tumors examined. This ranged from 0–1+ in 11 cases, 2+ in 13 cases, and 3+ in 7 samples. Fifteen (47%) of the 31 primary carcinomas showed intense staining in the epithelial cells of well-differentiated lesions with a glandular architecture (Fig. 1). The staining

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**Fig. 2** Intense staining of peripheral nerve with an antibody to TGF- $\alpha$ . Malignant epithelial cells are negative and adjacent to nerve fibers, suggesting a paracrine interaction.



pattern was more diffuse in higher grade neoplasms. In some high-grade lesions, a monolayer of immunoreactive tumor cells was intermixed with negatively stained stromal elements. Stromal cells were generally nonreactive for EGFr. Blood vessels were weakly positive. Half of the samples studied showed weak epithelial immunostaining for TGF- $\alpha$ , the rest were nonreactive. On consecutive sections, isolated cells were found to coexpress the ligand and the receptor. In contrast, stromal staining was intense for the ligand, including a strong immunoreactivity in peripheral nerves. We observed foci of TGF- $\alpha$ -unreactive malignant cells infiltrating TGF- $\alpha$ -positive nerves (Fig. 2).

#### Androgen-Independent Tumors

**Primary Lesions.** Three locally progressing tumors were available for study. All expressed EGFr in a diffuse pattern while TGF- $\alpha$  expression was undetectable on epithelial tumor cells.

**Metastases.** Of the 15 lymph nodes evaluated, 12 had adequate material for pathological evaluation. All were high grade. Overall, immunoreactivity for EGFr was observed in all 12 of the lymph nodes, all 5 of the hepatic specimens, as well as the 2 miscellaneous sites evaluated (Table 1). In all but 2 cases, the staining was 2+ or greater, consistent with a more homogeneous pattern of expression. Staining for the physiologically active EGFr ligand, TGF- $\alpha$ , was generally less intense within the tumor. Considering individual sites, positive TGF- $\alpha$ -immunoreactivity was observed in 8 (72%) of 11 nodal sites, 2 (40%) of 5 hepatic lesions, as well as the miscellaneous lesions studied.

Overall, coexpression of the ligand and the receptor by epithelial tumor cells was observed in 14 (78%) of the 18 hormone-refractory metastases evaluated. Of note was that all 14 metastatic lesions that expressed TGF- $\alpha$  coexpressed EGFr (Fig. 3):

#### DISCUSSION

The primary cause of prostate cancer death is metastatic disease that is refractory to androgen ablation. The clinical virulence of these tumors relative to early stage lesions, is shown by their ability to metastasize and to proliferate in a castrate environment. This shows that other factors, in addition to androgen, contribute to the growth of these cells. We postulated that direct characterization of these metastatic hormone-refractory tumors might lead to the identification of biological parameters that could lead to improved therapies.

The present study shows changing patterns of expression of EGFr and its physiological ligand, TGF- $\alpha$ , as prostate cancers progress from the primary hormone-naïve to a metastatic androgen-independent state. Although the immunohistochemical data do not permit an assessment of the functionality of the receptor *in vivo*, they do suggest a change from a predominantly paracrine relationship in the untreated primary to a potential autocrine interaction in androgen-independent metastatic lesions. The observation of TGF- $\beta$  negative malignant prostatic epithelial cells involving peripheral nerves that have a positive TGF- $\alpha$  phenotype in untreated primary tumors is consistent with a paracrine interaction (Fig. 2). The staining pattern for EGFr in

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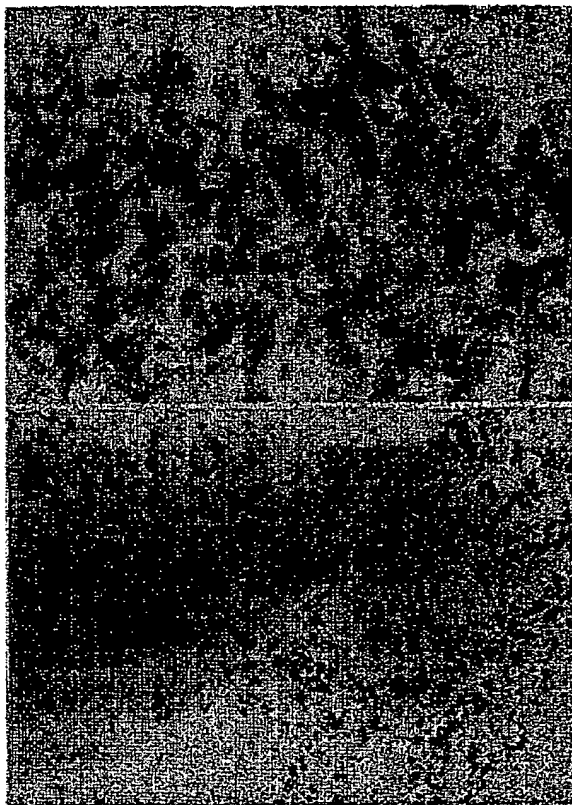


Fig. 3 EGFr expression in a hormone-refractory metastatic lesion. Note the diffuse staining pattern of the epithelial cells by both the EGFr (upper panel) and TGF- $\alpha$  (lower panel), suggestive of an autocrine loop.

malignant primary epithelial cells showed no association between immunoreactivity and grade. These findings are similar to those reported by others using an immunohistochemical evaluation of primary, hormone-naïve tumors (16, 27). In one study, however, although a glandular pattern of staining was predominant, 75% of the specimens evaluated showed no staining (14). TGF- $\alpha$  expression was predominantly stromal, although one half of the specimens evaluated showed staining on isolated epithelial tumor cells. This is similar to the results of Lloyd *et al.* (15) where 18 of 34 tumors evaluated showed staining and Harper *et al.* (21). In contrast, we found that metastatic lesions showed a more diffuse pattern of staining for EGFr, with coexpression of the receptor and the TGF- $\alpha$  ligand, in 78% of the cases studied.

The increase in staining observed in androgen-independent lesions could be attributed to the progressive increase in grade associated with metastatic progression (28). It may also be related to the metastatic process since the three androgen-independent relapsing primary tumors were negative. The data on the relationship between grade and EGFr expression are conflicting. Most studies reporting the association were based on tissue homogenates (13, 29) and not immunohistochemistry,

Table 1 Immunohistochemical results using mAb to the EGFr and TGF- $\alpha$  in androgen-independent metastases

Metastatic site	Score		EGFr	TGF- $\alpha$
Lymph nodes		No.	12	11
	3+		2	0
	2+		8	2
	0/1+		2	9 (1 Inev)
Liver		No.	5	5
	3+		1	0
	2+		4	0
	0/1+		0	5
Adrenal		No.	1	1
	2+		1	0
	0/1+		0	1
Bone		No.	1	1
	3+		1	0
	1+		0	1

which allows direct visualization of the cellular components where binding occurs. Two reports, one using the *in situ* hybridization technique for mRNA on paraffin-embedded material (17) and a second using immunohistochemistry, showed increased expression with increasing T stage ( $P = 0.007$ ) and higher grade ( $P = 0.012$ ). In contrast, Maddy *et al.* (30) observed decreased expression with increasing grade, while Ibrahim *et al.* (16) were unable to correlate grade with expression. They postulated that the low level of expression seen in some tumors may have been the result of more rapid turnover of the receptor, overexpression of a truncated version of the receptor as has been observed in malignant gliomas, down-regulation of the receptor, or possible proteolytic cleavage by a protease (16). The results do not exclude a possible relationship between metastatic potential and EGFr expression. Using human colon carcinoma cells with different malignant potential, Radinsky *et al.* (31) showed a direct relationship between EGFr expression and metastatic potential at the protein, RNA, and DNA levels. High EGFr-expressing cells had a higher rate of metastases. Furthermore, these cells were sensitive to levels of TGF- $\alpha$  found in regenerating liver. They postulated that TGF- $\alpha$ , released by the disruption of the normal cellular architecture when a metastasis occurs, could act in a paracrine fashion (31).

Several groups have shown that androgens up-regulate EGFr (32, 33) and promote EGF binding to malignant prostatic epithelial cells in culture (34). However, the high intensity of staining despite castrate levels of testosterone, coupled with the observed coexpression of the ligand and receptor, indicates an evolution to potential autocrine growth in androgen-independent disease in a significant proportion of cases. In addition, Bae *et al.* (35) showed that repeated passage of tumors in nude mice derived from human prostate epithelial cells immortalized by SV40 T antigen gene transfection decreased the latency period for tumor development. The decrease in latency was shown to be associated with activation of the TGF- $\alpha$ /EGFr autocrine pathway (35).

A limitation of this study was the unavailability of hormone-naïve metastases. Biopsies of these sites have not been routinely performed in our institution. Thus, the presence of the EGFr and its physiological ligand TGF- $\alpha$  in the evolution of androgen independence and its role in the metastatic process

requires further study. Recently, EGF has been shown to promote invasion (36) and to increase the metastatic diversity of prostate cancer cell lines (37). These effects, along with the data showing an effect of endogenous TGF- $\alpha$  and exogenous EGF on prostate cancer cell proliferation, suggest a diverse role for the EGFR and its ligands on prostate cancer cell growth. *In vitro*, antibodies to the EGFR inhibit the growth of prostate cancer cell lines (38, 39). These results, combined with the observed change in the pattern of expression of the receptor between hormone-naïve primary and androgen-independent metastatic lesions and the frequency of coexpression in metastatic lesions, suggest that interruption of this growth factor/growth factor receptor signaling pathway could be of therapeutic importance. The results also suggest the need to study each stage of prostate cancer progression separately, as different mechanisms may predominate as the disease evolves from the hormone-sensitive primary to the androgen-independent metastatic state.

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